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Isolation and characterization of coaggregation-defective (Cog$^-$) mutants of *Streptococcus gordonii* DL1 (Challis)

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***Streptococcus gordonii* DL1 (Challis) bears coaggregation-mediating surface adhesins which recognize galactoside-containing surface polysaccharides on *Streptococcus oralis* 34, *Streptococcus oralis* C104, and *Streptococcus* SM PK509. Fifty-nine spontaneously-occurring coaggregation-defective (Cog$^-$) mutants of *S. gordonii* DL1 unable to coaggregate with partner streptococci were isolated. Six representative Cog$^-$ mutants were characterized by their coaggregation properties with four *Actinomyces naeslundii* strains (T14V, PK947, PK606, PK984), *Veillonella atypica* PK1910, and *Propionibacterium acnes* PK93. The six representative Cog$^-$ mutants showed altered coaggregation with their streptococcal partners, *A. naeslundii* PK947, and *P. acnes* PK93. Based on the coaggregation phenotypes of these mutants, a model for the lactose-inhibitable coaggregation between *S. gordonii* DL1 and its partner bacteria is proposed. The potential use of these mutants in studies of oral biofilms is discussed.**

Keywords: coaggregation-defective mutants; accretion; oral streptococci; oral biofilms; adhesins

Introduction

Viridans streptococci constitute the majority of early colonizing bacteria on a clean tooth surface and represent between 60 and 80% of the cultivable cells found during the first 4 hours after professional teeth cleaning [17]. The remainder of the early colonizers are primarily actinomyces, veillonellae, and haemophili [18]. The oral (viridans) streptococci are unusual among the oral bacteria in that they also participate in extensive intrageneric coaggregation [4,12]. This property is shared to a much lesser extent by the oral actinomyces. Besides growth of the initally attached cells, bacterial repopulation of a cleaned tooth surface would be promoted by intrageneric coaggregation among streptococci and among actinomyces as well as by intergeneric coaggregation between streptococci and actinomyces. After the primary colonizers cover the tooth surface, further accretion of dental plaque may occur by intergeneric coaggregation involving other genera and the primary colonizers.

An interesting feature of coaggregations between and among oral bacteria is the fact that many coaggregations are inhibited by the addition of lactose [14]. In the context of using lactose to inhibit oral biofilm formation, the equal or greater number of coaggregations that are unaffected by lactose must be considered [10]. Furthermore, a given bacterial strain may possess and express multiple kinds of adherence-relevant surface molecules. One or more than one kind may function to mediate coaggregation between a given bacterial pair. To identify the potential surface protein that mediates coaggregation, it is advantageous to choose for study a coaggregating pair that interacts by a single kind of lactose-sensitive adhesin.

The goal of this study was to isolate coaggregation-defective (Cog$^-$) mutants unable to mediate lactose-inhibitable, intrageneric coaggregation among oral streptococci. *Streptococcus gordonii* DL1 (Challis) was chosen as the model to study intrageneric coaggregation. It has the heat- and protease-inactivated adhesin on the surface which recognizes galactoside-containing receptors on the surface of *Streptococcus oralis* 34, *Streptococcus oralis* C104, and *Streptococcus* SM PK509 [4,12]. In addition, *S. gordonii* DL1 can be made competent for the transformation of DNA. In this paper we report the isolation of 59 spontaneously occurring mutants of DL1 which fail to coaggregate with the streptococcal partners of DL1 and the further characterization of six of those mutants.

Materials and methods

Cultivation of bacteria

All bacterial strains are listed in Table 1. Species of *Streptococcus, Actinomyces*, and *Propionibacterium* were cultured in CAMG medium [15] at 37° C under anaerobic conditions with the GasPak system (BBL Microbiology Systems, Cockeysville, MD, USA). *Streptococcus gordonii* PK2975 was resistant to the antibiotics rifamycin (25 μg ml^{-1}), spectinomycin (500 μg ml^{-1}), and streptomycin (100 μg ml^{-1}) (Sigma Chemical Co, St Louis, MO, USA). Antibiotic resistance was obtained by plating a dense suspension of *S. gordonii* DL1 onto agar containing rifamycin, picking a colony and purifying by routine streak plating, and then repeating this procedure sequentially on agar containing spectinomycin and streptomycin. *Veillonella atypica* PK1910 was grown in a modified Schaedler's medium without glucose, but supplemented with 0.1 M sodium lactate [5].

Bacterial cells used for coaggregation assays were pel-

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Table 1 Strains used

Strain	Relevant characteristics	Reference or source
Streptococcus gordonii DL1 (Challis)	Reference strain for streptococcal coaggregation group 1	[10]
Streptococcus gordonii PK488	Reference strain for streptococcal coaggregation group 6	[10]
Streptococcus oralis 34	Reference strain for streptococcal coaggregation group 3	[10]
Streptococcus sanguis C104[a]	Reference strain for streptococcal coaggregation group 3	[10]
Streptococcus SM PK509	Reference strain for streptococcal coaggregation group 5	[10]
Streptococcus gordonii PK2975[b]	DL1 Rf^r Sm^r Sp^r	This study
Streptococcus gordonii PK1897[b]	PK2975 (Cog^-, selected with *S. sanguis* C104)	This study
Streptococcus gordonii PK3003[b]	PK2975 (Cog^-, selected with *S. sanguis* C104)	This study
Streptococcus gordonii PK3017[b]	PK2975 (Cog^-, selected with *S. sanguis* C104)	This study
Streptococcus gordonii PK3020[b]	PK2975 (Cog^-, selected with *S. sanguis* C104)	This study
Streptococcus gordonii PK3037[b]	PK2975 (Cog^-, selected with *S. sanguis* C104)	This study
Streptococcus gordonii PK3050[b]	PK2975 (Cog^-, selected with *S. sanguis* C104)	This study
Actinomyces naeslundii T14V	Reference strain for actinomyces coaggregation group A	[10]
Actinomyces naeslundii PK947	Reference strain for actinomyces coaggregation group C	[10]
Actinomyces naeslundii PK606	Reference strain for actinomyces coaggregation group D	[10]
Actinomyces naeslundii PK984	Reference strain for actinomyces coaggregation group E	[10]
Veillonella atypica PK1910	Reference strain for veillonella coaggregation group I	[5]
Propionibacterium acnes PK93	Lactose-inhibitable coaggregation with *S. gordonii* DL1	[2]

[a]Resistant to tetracycline (10 μg ml^{-1})
[b]Resistant to rifamycin (25 μg ml^{-1}), streptomycin (100 μg ml^{-1}), and spectinomycin (500 μg ml^{-1})

leted by centrifugation at 10 000 × *g* for 10 min at 4° C, washed three times in coaggregation buffer (1 mM Tris, pH 8.0, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 150 mM NaCl, and 0.02% NaN_3), and stored in coaggregation buffer at 4° C. The visual assay for coaggregation has been described in detail elsewhere [9,14].

Isolation of spontaneous Cog⁻ mutants

The antibiotic-resistant *S. gordonii* strain PK2975 was used to select Cog⁻ mutants by a method described earlier [7]. Briefly, after six cycles of mixing parent strain PK2975 with antibiotic-sensitive partner strain *Streptococcus oralis* C104, pelleting resultant coaggregates, and adding more partner cells to the supernatant fluid, the final supernatant fluid was serially diluted in CAMG broth and plated onto CAMG agar containing 25 μg rifamycin per ml of medium.

Results

By using failure to coaggregate with partner strain *S. oralis* C104 as the selection criterion, 59 spontaneously occurring mutants were isolated of which six were studied in more detail. The coaggregation properties of the wild-type strain *S. gordonii* DL1, the parent strain *S. gordonii* PK2975, and the six spontaneous Cog⁻ mutants are shown in Table 2. Both wild-type *S. gordonii* DL1 and the parent strain PK2975 showed nearly identical coaggregation patterns with *S. oralis* 34, *S. sanguis* C104, and *Streptococcus* SM PK509. As reported previously for the intrageneric coaggregations with strain DL1 [4,12], the intrageneric coaggregations with strain PK2975 were also reversed by adding lactose or *N*-acetyl-D-galactosamine (GalNAc). In all intrageneric coaggregations the *S. gordonii* strains expressed the heat and protease-inactivated surface components. The two *S. gordonii* strains differed, however, in their coaggregation patterns with the *Actinomyces* partners (Table 2). Both strains DL1 and PK2975 coaggregated in a nearly identical manner with *A. naeslundii* strains PK947 and PK606 and *V. atypica* PK1910. However, strain PK2975 did not coaggregate with *A. naeslundii* T14V and showed lactose-inhibitable coaggregation with *A. naeslundii* PK984. This difference in coaggregation between DL1 and PK2975 may be due to changes in the cell surface in the antibiotic-resistant mutant as has been reported by Jenkinson [6]. Additional studies of these differences have not been attempted.

All six representative spontaneous Cog⁻ mutants (strains PK1897, PK3003, PK3017, PK3020, PK3037, and PK3050) lost the ability to coaggregate with all three streptococcal partners and with the *A. naeslundii* strains T14V, PK947, and PK984 and *P. acnes* strain PK93 (Table 2). The lactose-noninhibitable coaggregations of the mutants with *A. naeslundii* PK606 and *V. atypica* PK1910 were essentially identical to the parent strain PK2975, indicating that the lactose-inhibitable coaggregations were specifically altered or lost.

Discussion

In this study we isolated and characterized spontaneous Cog⁻ mutants of *S. gordonii* DL1 that were unable to mediate lactose-inhibitable intrageneric coaggregation with their streptococcal partners (Table 2). Of the six mutants examined in detail, all possess the same phenotype. All of the mutants were isolated from a single selection procedure, and, thus, they may be progeny of the same original mutation. These Cog⁻ mutants simultaneously lost the ability to coaggregate with *A. naeslundii* PK947 and *P. acnes* PK93, which were also lactose-inhibitable coaggregations. Taken together, these data suggest that the abovementioned coaggregations are mediated by the same cell-surface adhesion on DL1 (Figure 1). Recently, Hsu *et al* [4] showed that coaggregation between *S. gordonii* DL1 and *S. oralis* 34 was inhibited most effectively by

Table 2 Coaggregation properties of spontaneous mutants of *S. gordonii* DL1 with several representative oral bacteria

Strain	Coaggregation score[a] with:								
	S. oralis 34	*S. oralis* C104	*Streptococcus* SM PK509	*A. naeslundii*				*V. atypica* PK1910	*P. acnes* PK93
				T14V	PK947	PK606	PK984		
Wild-type									
DL1	2^0	2^0	2^0	4^4	3^0	4^4	4^4	4^4	3^0
Parent									
PK2975	3^0	3^0	2^0	0	3^0	4^4	3^0	3^3	3^0
Cog⁻ mutants									
PK1897	0	0	0	0	0	4^4	0	3^2	0
PK3003	0	0	0	0	0	4^4	0	3^2	0
PK3017	0	0	0	0	0	4^3	0	3^2	0
PK3020	0	0	0	0	0	4^3	0	3^2	0
PK3037	0	0	0	0	0	3^3	0	3^2	0
PK3050	0	0	0	0	0	3^3	0	3^2	0

[a]The method for assigning coaggregation scores has been described by Kolenbrander and Andersen [9]. The maximum score is 4; no coaggregation is given a zero score. Coaggregation scores are given in two parts: the first score is that given after mixing the two strains together, and the superscript is the score after adding lactose (final concentration 60 mM) to the coaggregates

GalNAcβ1-3GalαOMe, which is part of the oligosaccharide repeating unit in the surface polysaccharide found on *S. oralis* strains 34 [16] and C104 [1].

An adhesin functionally similar to the one on *S. gordonii* DL1 may exist on the surface of *S. gordonii* PK488. Kolenbrander *et al* [12] showed that *S. gordonii* PK488 has the same streptococcal partners as *S. gordonii* DL1 (Figure 1). Spontaneous Cog⁻ mutants of *S. gordonii* PK488 unable to coaggregate with *S. oralis* C104 also are unable to coaggregate with *S. oralis* 34 and *Streptococcus* SM PK509 (Clemens and Kolenbrander, unpublished data, 1994). Mutants of *S. oralis* 34 unable to coaggregate with DL1 and PK488 still coaggregate with *Streptococcus* SM PK509 [14]. Thus, the adhesion on the surface of *Streptococcus* SM PK509 may be functionally less related to those found on the surface of *S. gordonii* strains DL1 and PK488.

S. gordonii strains [3,19] including DL1 [2] and PK488 (Ganeshkumar, personal communication, 1994) and *S. oralis* strains 34 and C104 [4] are all able to bind to salivary receptors present in the acquired pellicle on the tooth surface (Figure 1). After binding to the acquired pellicle, these cells then are able to coaggregate with other streptococci (intrageneric) as well as other genera of bacteria (intergeneric) to form a biofilm on the tooth surface. Bacteria that are unable to bind to the tooth surface directly, would have the opportunity to coaggregate with the already attached bacteria. Ciardi *et al* [2] showed that *P. acnes* PK93, which cannot bind directly to the tooth surface, coaggregates with *S. gordonii* DL1 that is bound to saliva-coated hydroxyapatite (SHA), a model of the tooth surface. Adherence of *P. acnes* PK93 to the SHA-associated DL1 is inhibited by GalNAc and lactose [2]. The *P. acnes*, which is bound to the surface-associated DL1, can then coaggregate with other bacteria forming a complex biofilm on the tooth surface. The complexity of this biofilm increases as more bacteria coaggregate with both the tooth surface-associated bacteria and the bacteria already bound to the tooth surface-associated bacterial layer [13].

The Cog⁻ DL1 mutants have lost the ability to mediate lactose-inhibitable intrageneric and intergeneric coaggregation but have retained the ability to mediate lactose-noninhibitable coaggregations (Table 2). As depicted in Figure 1, these mutants would still be able to attach to the acquired pellicle but would not show the extensive intrageneric coaggregations with partner streptococci as well as the intergeneric coaggregations with *A. naeslundii* PK947 and *P. acnes* PK93. The Cog⁻ DL1 mutants would still be able to coaggregate with *A. naeslundii* PK606 and *V. atypica* PK1910 (Table 2) as well as other oral bacteria that show lactose-noninhibitable coaggregation with DL1 [11,13,14]. In the context of the oral bacterial biofilm, there would be potential for a major change in the microbial community if the streptococci were unable to mediate lactose-inhibitable coaggregations. The Cog⁻ DL1 mutants could be used to study shifts in the microbial community using either SHA-associated Cog⁻ DL1 [2] or a flow cell containing a saliva-coated enamel surface [20], each bathed with a suspension of representative oral bacteria.

Although the wild-type *S. gordonii* DL1 and parent strain PK2975 exhibit the same coaggregations with streptococcal partners (Table 2), a dichotomy exists in their coaggregations with *Actinomyces* partners, *A. naeslundii* T14V and PK984 (Table 2). The coaggregation between DL1 and PK984 is not lactose-inhibitable (Table 2; [8]), however, the antibiotic-resistant mutant of strain DL1, parent strain PK2975, showed lactose-inhibitable coaggregation with PK984. The difference between DL1 and its antibiotic-resistant mutant, PK2975, may be due to changes in the cell surface. *S. gordonii* PK2975 is resistant to the hydrophobic antibiotic rifamycin (Table 1). Jenkinson [6] showed that a variant of *S. gordonii* (Challis) (formerly *S. sanguis* (Challis)) resistant to the hydrophobic antibiotic novobiocin showed a change in cell surface properties and a reduced degree of lactose-noninhibitable coaggregation with *A. naeslundii* (formerly *A. viscosus*) T14V and another *A. naeslundii* strain. In the present study, parent strain PK2975

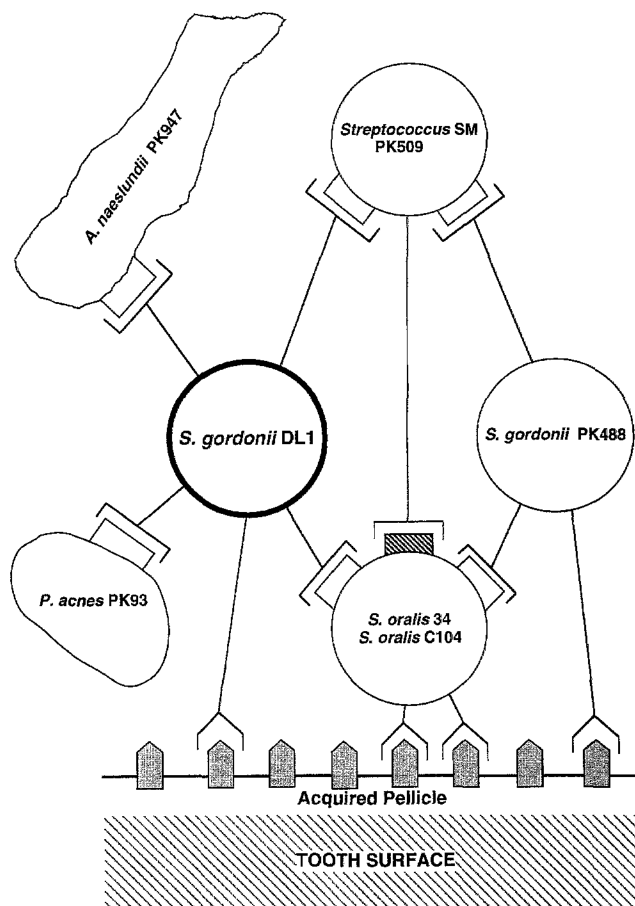

Figure 1 Diagrammatic representation of the lactose-inhibitable coaggregations between *S. gordonii* DL1 and its partners. Each interaction is depicted as a complementary set of symbols. The symbols with stems (called adhesins) represent heat- and protease-inactivated components on the surface of the respective stretococcal strains. The complementary rectangular symbols without stems (called receptors) represent components which are resistant to heat- and protease-treatment and which are thought to be galactoside-containing receptors. The striped rectangle on *S. oralis* 34 and *S. oralis* C104 is used to differentiate their coaggregation with *Streptococcus* SM PK509 from their coaggregations with *S. gordonii* DL1 and PK488 (open rectangle). The function of the four adhesins depicted as rectangles on stems on the surface of strain DL1 are simultaneously lost in the Cog⁻ DL1 mutants. *S. gordonii* strains DL1 and PK488 and *S. oralis* strains 34 and C104 bind to receptors in the acquired pellicle [2–4,19; Ganeshkumar, personal communication, 1994]. See text for details

also lost its lactose-noninhibitable coaggregation with *A. naeslundii* T14V (Table 2). The Cog⁻ mutants derived from PK2975 lost the lactose-inhibitable coaggregation with PK984 (Table 2). These data suggest that there may be both a lactose-inhibitable and a lactose-noninhibitable adhesin on strain DL1 mediating coaggregation with strain PK984 and just a lactose-noninhibitable adhesin mediating coaggregation with strain T14V. The rifamycin-resistant mutant of DL1, strain PK2975, may have lost the lactose-noninhibitable adhesin making the coaggregation between strains PK2975 and PK984 lactose-inhibitable.

Thus, this study has shown that intrageneric coaggregations among *S. gordonii* DL1 and other streptococci as well as intergeneric coaggregations between streptococci and actinomyces or propionibacteria may be mediated by the same adhesin on *S. gordonii* DL1. This adhesin is lactose-inhibitable and its function of mediating coaggregations with three genera of oral bacteria has been lost simultaneously. These data point at the interrelationship of two functions of adhesins: 1) to accrete bacteria and 2) to provide access to metabolic communication among adherent bacteria in the biofilm. The sharing and exchange cf metabolites as well as genetic information in the oral biofilm is a research area that has yet to be developed.

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